STUDIES ON OXIDATION AND REDUCTION BY PNEUMOCOCCUS.

I. Production of Peroxide by Anaerobic Cultures of Pneumococcus on Exposure to Air under Conditions Not Permitting Active Growth.

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INTRODUCTION.

The previous studies by McLeod and Gordon (1) and by Avery and Morgan (2) on the production of peroxide by pneumococcus have dealt with the formation and accumulation of this substance during active aerobic growth. The present paper presents a study of the production of peroxide by cultures in which its formation has been prevented during active growth, and in which the subsequent production of this compound could be followed under conditions unsuitable for the initiation and maintenance of cell reproduction. The cultures of pneumococcus employed in the following experiments were grown anaerobically in order to obtain bacterial cells not previously exposed to the action of the peroxide which may accumulate in the culture fluid during aerobic growth. Anaerobiosis was utilized, therefore, simply as a means of obtaining peroxide-free cultures in which could be studied the influence of certain factors on the potential ability of the cells to form peroxide when subsequently exposed to molecular oxygen.

The Formation of Peroxide by Anaerobic Cultures of Pneumococcus on Exposure to Air.

It was first desired to determine whether cells which had been grown anaerobically, that is under conditions precluding the formation of peroxide, would form this substance when subsequently exposed to air.

Cultures of Pneumococcus Type II were grown under vaseline seal in Florence flasks filled to the neck with broth. These cultures were incubated at 37°C. for 15 to 24 hours, which represent periods considerably beyond the phase of active growth. The anaerobic cultures were then cooled to 15°C. in running water; 5 cc. portions of broth culture were removed and placed in flasks of 300 cc. capacity. The culture fluid was then thoroughly aerated by shaking the flask for various lengths of time at room temperature. At intervals the culture fluid was tested for the presence of peroxide.[1]

Experiments of this nature have definitely shown that the bacterial cells, which have been grown under conditions precluding peroxide production, rapidly form peroxide when shaken in the air. Under these conditions peroxide in many instances may be demonstrated at the end of 5 minutes aeration. That the peroxide thus formed does not represent the oxidation of a metabolite free in the culture fluid, is shown by the fact that the supernatant fluid of centrifuged young anaerobic cultures remains peroxide-free after long periods of shaking in the air.

Influence of Age of the Culture upon the Formation of Peroxide by Anaerobically Grown Pneumococci When Exposed to Air.

The following experiment was conducted to test the influence of age and condition of the cell upon the ability of anaerobically grown pneumococci to form peroxide on exposure to air.

Two Florence flasks were completely filled to the neck with freshly boiled broth, sealed with vaseline, and inoculated each with a different strain of Pneumococcus

[1] Peroxide tests were made as follows: 0.5 cc. of the liquid to be tested was placed in a small agglutination tube. A small piece of freshly cut potato was introduced to serve as peroxidase. The presence of peroxide was then detected by the addition of 2 drops of a saturated solution of benzidine in glacial acetic acid. This test gave faint but definite reactions with broth containing 0.002 per cent of H_2O_2. By maintaining constant all conditions of the test, some idea of the comparative amount of peroxide can be estimated by the intensity of the reaction when compared with broth to which known amounts of titrated H_2O_2 had been added immediately before testing. The peroxidase-benzidine test is not specific for H_2O_2 and positive reactions would probably be given by any peroxide which can be split by the peroxidases of the potato. However, the fact that the peroxide which finally accumulates in pneumococcus cultures is hydrogen peroxide, has been proved by McLeod and Gordon.

Type II. After 15 hours incubation at 37°C., 15 cc. portions of each culture were transferred to a number of narrow test-tubes and sealed with vaseline. These anaerobic cultures, which had received the same initial seeding, were then allowed to age at 37°C. After periods indicated in Table I, the anaerobic cultures, free from peroxide, were exposed to air[2] in 150 cc. Erlenmeyer flasks at room temperature in the dark. The aerated cultures were tested for the presence of peroxide at frequent intervals during the period of exposure to air. The results of this experiment are condensed in Table I.

TABLE I.

Influence of Age of Cells upon the Rate and Persistence of Peroxide Formation by Anaerobically Grown Pneumococcus on Exposure to Air.

Age of anaerobic culture before exposure to air. (Pneumococcus Type II.)	Peroxide formation after exposure to air at 20°C.			
	Time of appearance.		Persistence after 10 days exposure to air.	
	Strain R.	Strain G.	Strain R.	Strain G.
hrs.	*hrs.*	*min.*		
15	4	30	+*	+
days				
2	6	30	−	+
3	6	30	−	+
4	6	30	−	+
6	8	30	−	−
		hr.		
15	†	1	−	−
70	‡	1	−	−

* In this table − indicates negative peroxide reaction; +, positive peroxide reaction.

† No peroxide detected.

‡ Faint traces of peroxide after 1 hour's exposure to air; the presence of detectable amounts was very transient, and only negative tests were obtained after 2 hours.

[2] Unless otherwise stated, cultures were "exposed to air" under the following conditions. The peroxide-free anaerobic cultures were shaken vigorously with air for 5 minutes. After preliminary agitation the aerated cultures were further exposed to molecular oxygen by placing the fluid in shallow layers at room temperature in the dark. The actual rate of peroxide production under these conditions is limited by the diffusion of oxygen into the non-agitated liquid. However, the preliminary, violent agitation, the shallow layers of liquid exposed, and the maintenance of constant conditions throughout all experiments, tend to minimize this objection in comparative studies of any given series.

The second and third columns in the table record the length of time required for the various cultures to produce a detectable amount of peroxide when exposed to the air. Hence, these figures in these columns give information concerning the rate of peroxide production by cells of different ages.

Peroxide is not a stable substance in broth and, even in the absence of catalase, it is gradually destroyed in this medium. Hence, the length of time the peroxide persists in the above tests will depend upon the actual amount of peroxide produced and upon the ability of the cells to continue its formation. The data presented in the fourth and fifth columns, therefore, serve as an index of the relative amount of peroxide produced by the cells of different ages, and of their ability to continue the production of peroxide.

It is apparent (Table I) that the "age" of the cultures at the time when the cells are exposed to oxygen affects the rate with which peroxide is formed. With increasing age of the anaerobic cultures there tends to be a gradual decrease of the potential peroxide-forming power of the cells. Nevertheless, it is evident that cultures which have passed beyond the phase of active growth and in which cell death and autolysis are occurring are still able to form peroxide when exposed to air.

It was thought that the storage of the mature anaerobic culture at low temperature would slow down the processes involved in the "aging" of the cells, and that, consequently, cultures held at low temperature would exhibit a more gradual loss in their ability to form peroxide. To investigate this question, the above experiment was performed in duplicate series, in which 15 hour anaerobic cultures were "aged" at 2° and at 37°C. Contrary to the results anticipated, no differences were observed in the effect of these two temperatures upon the ability of the anaerobically grown cells to form peroxide when exposed to molecular oxygen.

Table I also shows that marked differences exist in the readiness with which peroxide is formed by cells of different strains of pneumococcus. The shorter time required for production of detectable traces of peroxide shows that this process is much more rapid in young cultures of Strain G, than in those of Strain R. This difference in activity of peroxide production increases with the age of the cells. Strain R shows a gradual diminution of peroxide formation; Strain G, on the other hand, not only formed larger amounts of peroxide but its activity is less influenced by age than is that of the other strain.

That the more pronounced peroxide formation by Strain G is not related to viability or longevity was demonstrated by the fewer number of colonies developing on subculture.

Peroxide Formation by Anaerobically Grown Pneumococci When Exposed to the Air at Different Temperatures.

The ability of anaerobically grown pneumococci to produce peroxide when exposed to the air at different temperatures was tested in the following experiment.

TABLE II.

Peroxide Formation by Anaerobically Grown Pneumococci When Exposed to Air at Different Temperatures.

Period of exposure to air.	Strain R exposed to air at.			Strain G exposed to air at.		
	2–4°C.	20°C.	37°C.	2–4°C.	20°C.	37°C.
min.						
0	−	−	−	−	−	−
30	−	−	−	−	+	+
hrs.						
1	−	−	+	−	+	+++
2	−	−	++	−	+++	+++
4	−	+	++	+	+++	+++
6 and 9	−	++	++	++	+++	+++
days						
1, 2, and 3	−	++	++	++	+++	+++
5	−	+	+	++	+++	+++
10	−	±	±	++	+++	+++

In Tables II to IV − indicates that no peroxide was detected; ±, faint peroxide reaction; +, weak peroxide reaction; ++, moderate peroxide reaction; +++, marked peroxide reaction; and ++++, strong peroxide reaction.

15 cc. portions of the anaerobic cultures used in the preceding experiment were transferred to narrow test-tubes and overlaid with vaseline. The sealed cultures were placed in water baths at 2°, 20°, and 37°C. After they were brought to the desired temperature, the peroxide-free cultures were transferred to 150 cc. Erlenmeyer flasks and exposed to air. The respective temperature conditions were maintained throughout the manipulations. Peroxide tests were made at frequent intervals, the results of which are given in Table II.

Analysis of Table II shows that the maximum capacity to form peroxide was exhibited by pneumococcus cells aerated at 37°C. The

peroxide production by Strain G is again more pronounced and its activity is also much more independent of temperature than is that of Strain R. When the exposure to oxygen is carried out at 20°C., differences between the two strains become more marked. In the case of Strain R, an oxygen exposure of 4 hours is necessary before the appearance of peroxide in the culture. At a temperature of 2–4°C. Strain G shows a marked diminution in rate of peroxide production, while in the case of Strain R no peroxide was detected at any time during an observation period of 10 days. The fact that peroxide is formed by Strain G at 2°C. is evidence that this process may proceed at temperatures incompatible with cell multiplication.

Peroxide Production by Anaerobic Cultures of Pneumococcus When Readjusted to Different pH and Exposed to the Air.

It was desired to test the ability of anaerobically grown pneumococci to produce peroxide in culture fluid which, prior to aeration, had been readjusted to different hydrogen ion concentrations.

A broth culture of Pneumococcus Type II (Strain G) was grown in plain broth under anaerobic conditions for 15 hours at 37°C. At the end of the period of incubation the culture fluid, containing the anaerobically grown cells, was readjusted to pH zones 4 to 8.5 by the addition of N HCl and N NaOH. Under these conditions the organisms were subjected to the influence of these various reaction changes for 20 minutes before aeration. The readjusted cultures were then transferred to 150 cc. Erlenmeyer flasks and exposed to the air at room temperature. Tests were made at different intervals during aeration to determine the time of appearance of peroxide. The results are given in Table III.

Cultures in which the formation of peroxide has been precluded by the exclusion of air during growth may be subjected to reaction changes covering a wide range of H ion concentration without loss of their ability to form peroxide when subsequently exposed to the action of molecular oxygen (Table III).

Although the maximum production of peroxide occurs between pH 6.3 and 8.5, small amounts of peroxide may be produced at reactions approaching pH 5.0. That peroxide does not continue to be produced at this reaction is indicated by its gradual disappearance from the medium. At reactions more acid than pH 5.0 (as pH 4.0), no peroxide was formed even after prolonged aeration.

As shown in previous experiments, peroxide may be produced in cultures in which active growth has ceased and at temperatures outside the range of growth possibility. Likewise, in the present experiment, it has been found that the pH zone within which peroxide can be formed is considerably extended both on the acid and alkaline side beyond that which limits growth. In fact, the formation of small amounts of peroxide at pH 5.0 indicates that this process can be initiated at a pH which marks the acid death-point of pneumococcus.

TABLE III.

Peroxide Formation by Anaerobic Cultures of Pneumococcus When Readjusted to Different pH and Exposed to the Air.

pH to which culture was readjusted.	Peroxide formation after exposure to air for.				
	0	30 min.	2 hrs.	1 day.	4 days.
4.0	−	−	−	−	−
5.0	−	+	++	±	−
6.3	−	+	+++	++++	++++
7.1	−	+	+++	++++	++++
8.4	−	+	+++	++++	++++

Formation of Peroxide by Heated Anaerobic Cultures of Pneumococcus on Exposure to Air.

The following experiment was planned to determine whether or not anaerobically grown pneumococci which have been heated at 50°C. for varying periods of time, can form peroxide when subsequently exposed to air.

A 15 hour anaerobic culture of pneumococcus (Strain G) was prepared as described before. 4 cc. portions of this culture were placed in agglutination tubes and sealed with vaseline. These were then heated in a water bath at a constant temperature of 50°C. At stated intervals, duplicate tubes were removed and immediately cooled to 15°C. The contents of these tubes were then placed in 100 cc. Erlenmeyer flasks and exposed to the air at room temperature in the dark. Sterility tests of the heated cultures were made by plating 0.1 cc. on blood agar and inoculating 0.2 cc. in blood broth. Results are given in Table IV.

It is evident (Table IV) that, in the case of the strain of pneumococcus studied, the properties of the cell upon which peroxide for-

mation depends are thermolabile, and are destroyed by exposure to 50°C. for 15 to 30 minutes. Cultures which had been heated for 10 minutes at 50°C., but which contained a few viable cells, still retained the property of forming peroxide, in appreciable quantities, when subsequently exposed to the air. After 15 minutes exposure to this temperature, no viable cells could be determined by cultural methods, although significant, but transient, traces of peroxide were demonstrable after aeration.

TABLE IV.

Formation of Peroxide by Heated Anaerobic Cultures of Pneumococcus on Exposure to Air.

Anaerobic cultures heated at 50°C.	Peroxide tests after exposure of cultures to air at 20°C.				Presence of viable cells in culture tests after heating.
	0	1 hr.	2 hrs.	24 hrs.	
Unheated.	−	+++	+++	+++	+
min.					
5	−	+	++	++	+
10	−	+	++	+	+
15	−	±	±	−	−
30	−	−	−	−	−
60	−	−	−	−	−

DISCUSSION.

When cultures of pneumococci are grown anaerobically, and subsequently exposed to air, peroxide can be readily detected in the culture fluid. The formation of peroxide under these conditions is prompt, the reactive substances of the cells in the presence of molecular oxygen giving rise to demonstrable amounts of this compound after brief aeration. Although the property of forming peroxide was exhibited by all pneumococci, certain variations in degree of this activity and in the susceptibility of this process to environmental conditions were observed among different strains. Moreover, the property of the bacterial cell, upon which the formation of peroxide depends, is to some extent affected by the length of time the culture is held under anaerobic conditions before the cells are brought into contact with air. However, cultures, in the absence of molecular oxygen, still retain potentially reactive substances only slightly

impaired by the processes of aging when compared with the marked cellular changes which accompany senescence. Cell disintegration and autolysis, which always mark the cessation of growth of pneumococcus, have but little effect upon the avidity with which the cellular substances react with oxygen to form peroxide.

Cultures grown anaerobically, and then subjected in the absence of air to reaction changes covering a wide range of H ion concentration, pH 5.0 to 8.5, still possess the property of forming peroxide when subsequently exposed to the action of molecular oxygen. This range of H ion concentration is much wider than that within which cell growth can be initiated, and even includes the acid zone in which cell death occurs. Although temperature influences the rate of peroxide production, this property is exhibited by cultures exposed to temperatures much lower than those compatible with cell reproduction.

SUMMARY.

1. Anaerobically grown pneumococci rapidly form peroxide upon exposure to molecular oxygen.

2. The peroxide-forming activity of pneumococci varies with different strains, and with the age of the cell.

3. Peroxide production by pneumococcus can proceed under conditions of reaction and temperature that do not permit active growth and multiplication of the cell.

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